INTERLEUKIN-1β CONVERTING ENZYME INHIBITION BLOCKS PROGRESSION OF TYPE II COLLAGEN-INDUCED ARTHRITIS IN MICE

George Ku, Ted Faust, Linda L. Lauffer, David J. Livingston, Matthew W. Harding

To IL-1β is a principal mediator in the pathogenesis of inflammatory disease. The IL-1β-converting enzyme (ICE), a novel cysteine protease, is required for processing of the 31 kDa IL-1β precursor to generate the 17 kDa proinflammatory mature form. We investigated the effect of two irreversible peptidyl ICE inhibitors, VE-13,045 and VE-16,084, on IL-1 production in vitro and in vivo in acute and chronic inflammatory disease models. In vitro, VE-13,045 and VE-16,084 inhibited IL-1β secretion by LPS-stimulated human adherent mononuclear cells (IC_{50}'s of 0.4 μM and 2.0 μM, respectively) and murine splenic monocytes (IC_{50}'s of 10 μM and 1.3 μM, respectively). Both VE-13,045 and VE-16,084 also inhibited LPS stimulated IL-1α secretion, although with reduced potency. In vivo, a single intraperitoneal dose of VE-13,045 (50 mg/kg) administered to mice 60 to 75 minutes after a 40 mg/kg LPS challenge significantly reduced IL-1β serum levels by 50 to 70%. In the DBA/1J mouse model of Type II collagen-induced arthritis, prophylactic treatment with VE-13,045 (50 and 100 mg/kg/day) significantly delayed the onset of inflammation, with a 60% overall reduction in disease severity. VE-13,045 was more effective than either indomethacin (2 mg/kg/day) or methyl prednisolone (10 mg/kg/day). VE-13,045 was also effective in reducing inflammation and progression of arthritis when administered to mice with established disease. Histological analysis of wrist joints showed a reduction in synovial membrane damage, inflammatory cell infiltration and fibrosis, and cartilage erosion in VE-13,045-treated animals. This is the first demonstration of efficacy for an ICE inhibitor in a chronic disease model and suggests that ICE is an important target for design of anti-inflammatory or disease modifying drugs.

IL-1 is a principal mediator in the pathogenesis of several diseases including rheumatoid arthritis, systemic inflammatory response syndrome (SIRS), inflammatory bowel disease, glomerulonephritis and insulin-dependent diabetes mellitus.[1] The proinflammatory activities of IL-1 have been characterized extensively and its importance in the pathogenesis of multiple diseases reflects IL-1 induction of other cytokines, cell adhesion molecules and inflammatory mediators.[2,3] There are two IL-1 agonists, IL-1α and IL-1β, that share minimal sequence identity;[4,5] however, both IL-1α and IL-1β interact with the same cellular receptors and their biological activities are indistinguishable.[6,7] Several strategies for blocking IL-1 have been explored, including use of the IL-1 receptor antagonist (IL-1RA) and soluble IL-1 receptors (sIL-1R).[8,9] Results from animal and initial human studies show that blocking IL-1 activity may have a therapeutic benefit. In animal studies, IL-1RA or sIL-1R treatment reduces the severity of endotoxin induced sepsis,[10-12] prevents allograft rejection,[9] graft versus host disease[13] and blocks progression of arthritis in experimental models.[14-16] Initial clinical studies in patients with rheumatoid arthritis suggests that intra-articular or subcutaneous administration of sIL-1R or IL-1RA may reduce joint tenderness and inflammatory symptoms.[17-19] However, in patients with SIRS, IL-1RA treatment has a more limited therapeutic benefit, which may reflect the involvement of TNF-α and other mediators.[20]

IL-1β and IL-1α are each synthesized as 31-33 kDa precursors lacking conventional secretory signal sequences[4,5] and both cytokines are processed to mature forms by proteases.[21-24] Membrane associated IL-1α precursor has biological activity,[25,26] although the IL-1α precursor may be processed to its mature form by the calcium-dependent protease, calpain.[21,22] Mature 17 kDa IL-1β is generated by the IL-1β converting enzyme

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(ICE), a unique cytoplasmic cysteine protease that is essential for IL-1β precursor processing and export of mature IL-1β from monocytes.[27,28] ICE is composed of two non-identical 20 and 10 kDa subunits which are derived from a 45 kDa proenzyme by autoprocessing.[27,29] The three-dimensional structure of ICE complexed with prototype inhibitors has been solved and catalytic residues in the active site have been identified.[30,31]

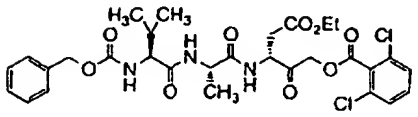
There is considerable interest in ICE as a target for design of novel anti-inflammatory or disease modifying drugs. Studies with prototype ICE inhibitors have demonstrated activity in blocking IL-1β production by monocytes in vitro and in whole blood assays.[27] However, the potential of ICE inhibitors for efficacy in vivo has not been evaluated. We have initiated experiments to investigate the biological function of ICE in vivo, including studies with ICE-deficient mice.[32] Here we have utilized two prototype peptidyl ICE inhibitors, VE-13,045 (carbobenzyloxy-Val-Ala-Asp-(O-Et)-CH_2O-dichlorobenzoate), and VE-16,084 (carbobenzyloxy-Val-Ala-Asp-CH_2O-dichlorobenzoate) to evaluate the efficacy of ICE inhibition on IL-1 production in vivo. In a model of SIRS, we measured the effect of ICE inhibition on IL-1β serum levels in LPS-challenged mice. We chose Type II collagen-induced arthritis (CIA) in the mouse as a model of rheumatoid arthritis (RA). CIA involves MHC class II linkage, humoral and cellular immunological responses to Type II collagen (CII), synovial inflammation, cartilage and bone destruction similar to human RA.[33] Neutralization of IL-1 activity in vivo with anti-IL-1β and anti-IL-1α antibodies or IL-1RA blocks disease progression[34,35] suggesting a pre-dominant role for IL-1 in the pathogenesis of CIA. We examined, therefore, the therapeutic potential of ICE inhibitors in this chronic disease model.

RESULTS

Chemical structure and properties of prototype peptidyl ICE inhibitors

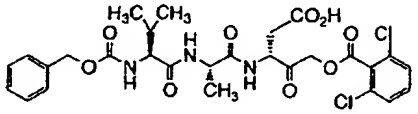
VE-13,045 and VE-16,084 (carbobenzyloxy-Val-Ala-Asp-(O-Et)-CH_2O-dichlorobenzoate and carbobenzyloxy-Val-Ala-Asp-CH_2O-dichlorobenzoate; Figure 1) are potent irreversible ICE inhibitors. In an in vitro enzyme assay, VE-13,045 and VE-16,084 inhibit ICE activity with IC_{50}'s of approximately 1 μM and 10 nM, respectively. The two compounds differ only by the presence of an ethyl ester side chain in the aspartyl moiety of VE-13,045. In cellular assays in vitro, the ester side chain is probably hydrolysed to the free acid (VE-16,084) by membrane non-specific acid esterase. Differences in potency observed with these compounds

VE-13,045



carbobenzyloxy-Val-Ala-Asp(OEt)-CH_2O-dichloro-benzoate

VE-16,084



carbobenzyloxy-Val-Ala-Asp-CH_2O-dichloro-benzoate

Figure 1. Chemical structure of prototype peptidyl ICE inhibitors, VE-13,045 and VE-16,084.

in vitro may reflect plasma membrane lipid composition and permeability, enhanced or diminished uptake of the ester or acid forms, rate of ester hydrolysis to the acid, and general stability of the compounds after exposure to cellular proteases. In vivo, the aspartyl ester side chain in VE-13,045 is rapidly hydrolyzed, with a serum half life of about 10 min, yielding VE-16,084 (I.R. Ager and J. M. C. Golec, personal communication) as the bioactive molecule.

Inhibition of ICE activity in vitro blocks secretion of both IL-1β and IL-1α

VE-13,045 and VE-16,084 block secretion of IL-1β (IC_{50}'s of 0.4 μM and 2.0 μM, respectively) by LPS stimulated human adherent monocytes (Fig. 2A). Both compounds also block IL-1β secretion after nigericin treatment[36] of murine splenic monocytes stimulated overnight with LPS. Here, VE-16,084 is more potent (IC_{50} ~1.3 μM) than VE-13,045 (IC_{50} ~10 μM; Fig. 2B). The cellular potency of VE-16,084 for inhibition of IL-1β secretion by murine splenic monocytes is consistent with results from an independent study with the same compound (WIN 67694) after LPS stimulation of thioglycolate elicited murine peritoneal macrophages.[38]

Surprisingly, VE-13,045 and VE-16,084 block secretion of IL-1α by human adherent monocytes (IC_{50}'s of 0.3 μM and 10 μM, respectively; Fig. 2A). Both compounds also affect IL-1α secretion by murine splenic monocytes treated with nigericin, however, VE-13,045 is a less potent inhibitor of murine IL-1α secretion (IC_{50} of >20 μM) than VE-16,084 (IC_{50} ~10 μM; Fig. 2B). No inhibition of TNF-α or IL-6 secretion was observed with VE-13,045 or VE-16,084 in these experiments (not shown). Also, VE-13,045 and VE-16,084 did not effect viablity of human or mouse mono-

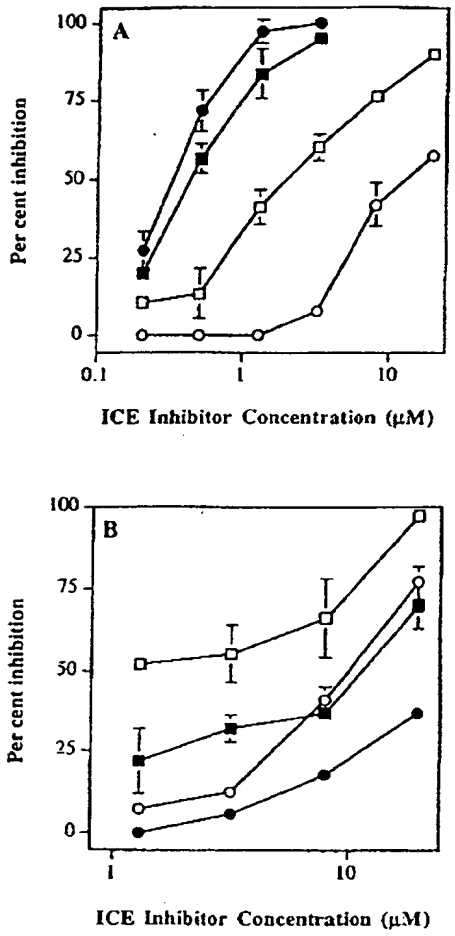


Figure 2. Effect of VE-13,045 and VE-16,084 on IL-1β and IL-1α secretion by LPS-stimulated (A) human adherent monocytes and (B) nigericin treated murine splenic monocytes.

Human or murine cells were incubated with LPS (1 μg/ml and 10 μg/ml, respectively) for 16–18 h. Murine cells were then washed and incubated with 10 μM nigericin for an additional 30 min. Cytokine levels in culture supernatants were measured by ELISA. Results are expressed as the % inhibition (mean ± SEM) by VE-13,045 (closed symbols) or VE-16,084 (open symbols) for IL-1β (squares) and IL-1α (circles) secretion compared to LPS-stimulated control cultures. These data are representative of at least four replicate experiments.

cytes in these assays as measured by trypan blue staining or lactate dehydrogenase activity in culture supernatants, and concentrations as high as 40 μM have no effect on proliferation of THP-1 monocytes (not shown).

VE-13,045 blocks LPS-induced IL-1β production in vivo

Initial experiments established an LPS dose-response and time course profile of serum cytokines in CD1 mice. After administration of LPS at 40 mg/kg, serum IL-1α and IL-1β levels begin to increase after 1–2 h and peak at 6–8 h while serum TNF-α or IL-6 reached maximal levels at 1.5–2 h. VE-13,045 was administered before, with or after LPS and serum IL-1α and IL-1β levels were measured 7 h after LPS challenge. Significant inhibition of LPS-induced IL-1β production was observed with a single 50 mg/kg dose of VE-13,045 administered 60–75 min after LPS (Table 1). VE-13,045 administration 30, 60 or 90 min before or simultaneously with LPS failed to reduce IL-1β levels (data not shown). Some reduction in serum IL-1α levels was also noted in VE-13,045 treated mice, but this effect was variable. No effect of VE-13,045 was observed on serum levels of TNF-α or IL-6 in LPS-challenged mice (data not shown).

TABLE 1. Effect of VE-13,045 administration on IL-1b serum levels in LPS challenged CD1 mice

Time of VE-13,045 administration relative to LPS-challenge	Number of determinations	Serum IL-1β (pg/ml)	Mean % inhibition
LPS control	4	490 ± 164	
Concurrent with LPS	2	460 ± 35	7
+30 min	2	414 ± 216	15
+45 min	2	473 ± 264	4
+60 min	4	214 ± 153	56
			$P < 0.01$
+75 min	2	158 ± 63	68
			$P < 0.01$
+90 min	2	474 ± 67	4

CD1 mice (n = 6–10 per dose group) were challenged with LPS (40 mg/kg in 0.5% CMC-PBS at 20 ml/kg) by intraperitoneal injection. VE-13,045 (50 mg/kg) prepared in olive oil:ethanol:DMSO (90:5:5) was also administered by intraperitoneal injection simultaneously with LPS or at time points after LPS challenge. Mice were bled 7 h after LPS challenge and IL-1b serum levels were determined by ELISA.

This effect of the timing of VE-13,045 administration on reduction of IL-1β serum levels is consistent with its pharmacokinetics and the time course of IL-1β production. In pharmacokinetic studies, a single 20 mg/kg intraperitoneal dose of VE-16,084 in mice resulted in a maximum plasma concentration (C_{max}) of approximately 10–12 μg/ml within 10–15 min and an estimated terminal elimination half life ($t_{1/2}\beta$) of 15–20 min (I. R. Ager and J. M. C. Golec, unpublished observations). Therefore, a maximum inhibitory effect was observed when plasma drug concentrations coincided with the initial increase in IL-1β production (between 1–2 h), resulting in maximum reduction in the serum IL-1β levels measured at the 7 h time point.

Incidence and progression of Type II collagen-induced arthritis

We initially evaluated the incidence, severity and progression of Type II collagen arthritis in DBA/1J mice

induced by immunization with 100 μg CII followed by a booster injection of 100 μg CII 21 days later.[34,37] We detected a 60% incidence of arthritic disease when following this protocol, with an average severity of level 2 (focal swelling of the wrist) observed within 14 days of the CII booster injection. When the booster injection was increased to 200 μg CII, we detected a 100% incidence of inflammation and a consistent time course progression of arthritic disease. Erythema (level 1) was first evident 4-5 days after the CII booster injection. Focal carpal (wrist) joint swelling (level 2) occurred within 8-12 days, with progressive swelling of the entire wrist (level 3) by day 16 to 18. Progression of swelling to the metacarpal/metatarsals (palm region; level 4) was observed in 10-15% of the animals and further progression to the metacarpophalangeal or metatarsalphalangeal joints (level 5) in only 5% of the animals.

Prophylactic and therapeutic efficacy of VE-13,045 in Type II collagen-induced arthritis

The incidence and progressive pattern of inflammation and arthritic disease observed in the modified Type II collagen arthritis model described above appeared to be suitable for testing the therapeutic potential of ICE inhibitors. Therefore, we evaluated

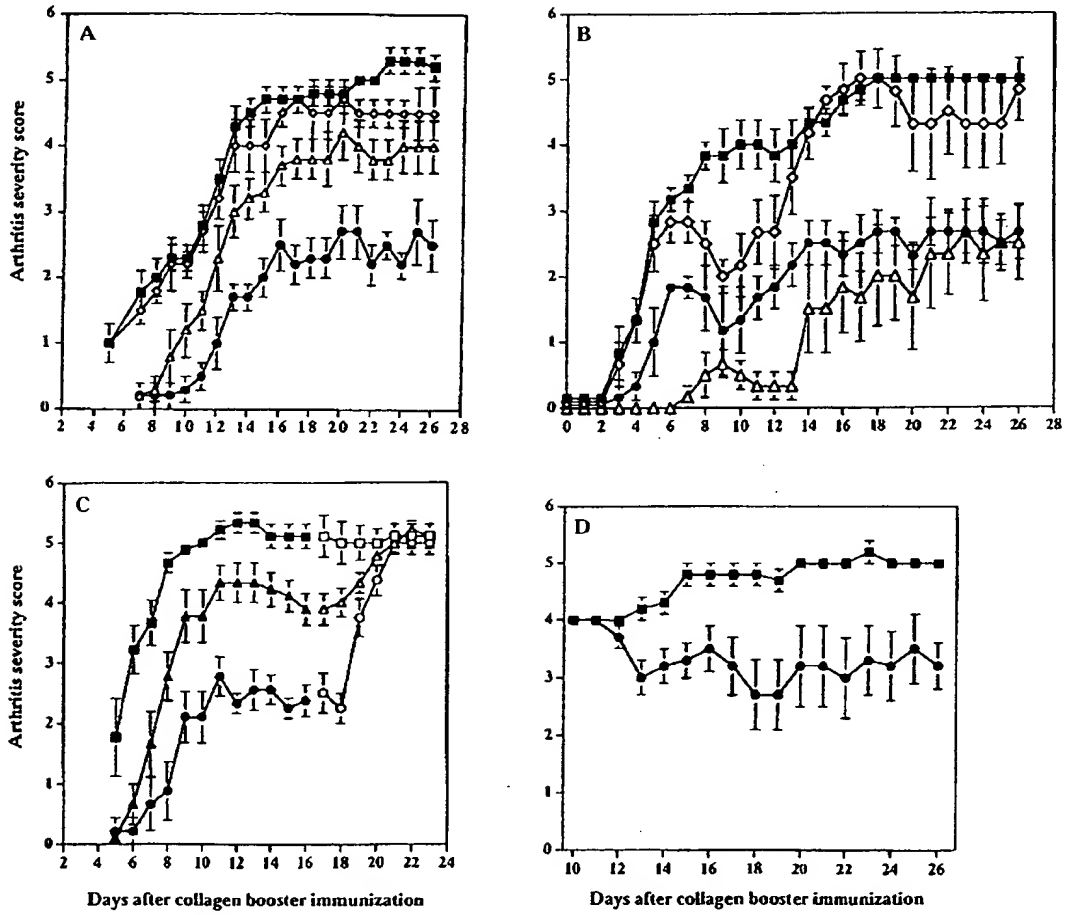


Figure 3. Effect of VE-13,045 on the clinical course of Type II collagen-induced arthritis in DBA/1J mice.

(A) Prophylactic therapy with VE-13,045 (●; 50 mg/kg/d), prednisolone (△; 10 mg/kg/d) and indomethacin (◇; 2 mg/kg/d) compared to vehicle-treated mice (■). (B) Prophylactic dose-response study with VE-13,045 at 100 mg/kg/d (△), 50 mg/kg/d (●) and 25 mg/kg/d (◇) compared to vehicle-treated mice (■). (C) Prophylactic therapy with VE-13,045 (●) and prednisolone (▲) followed by treatment termination (open symbols). (D) Clinical course of established CIA in VE-13,045 treated (●) or vehicle-treated (□) mice starting 10 days after the CII booster injection. Mice (6-10 per group) were injected intradermally with 100 μg of chick CII on day 0, followed by a 200 μg booster injection on day 21. Front paws were examined daily after the CII booster injection, the status of arthritic disease was scored as described in Methods, a combined score of front paws was determined and expressed as the mean ± SEM for each treatment group.

VE-13,045 for prophylactic efficacy in comparison to indomethacin and methyl prednisolone. Results in Figure 3A demonstrate superior efficacy of VE-13,045 in reducing the severity of inflammation and progression of arthritic disease. Daily administration of VE-13,045 (50 mg/kg) delayed the onset of inflammation by 6 days with a 60% overall reduction in arthritic disease compared to vehicle-treated animals (significance of $P < 0.01$ for days 10 to 26). In contrast, daily administration of indomethacin (2 mg/kg) failed to prevent the progression of arthritis, whereas methyl prednisolone (10 mg/kg) delayed the onset of inflammation by 4 days, but reduced overall disease severity by only 20% (significance of $P < 0.05$ for days 18–26; Fig. 3A).

Another experiment (Fig. 3B) tested VE-13,045 doses of 25, 50 and 100 mg/kg/day. Treatment with 100 mg/kg delayed onset of inflammatory symptoms by 10 days compared to untreated animals. The progression of disease in this group was slower between days 15 and 20, however, after 22–26 days, the severity of arthritic disease was similar to the 50 mg/kg treatment group, with a combined severity score of approximately 2.5 for both groups. VE-13,045 treatment at 25 mg/kg/day had a transient effect on symptoms (between days 7 to 13), but no overall sustained or consistent effect on progression of arthritic disease.

In another experiment, animals were treated with VE-13,045 (50 mg/kg) or prednisolone (10 mg/kg) for 16 days and then treatment was discontinued. The degree of inflammation in the affected paws increased within 2–3 days and the severity of arthritic disease rapidly progressed to the same level as the vehicle treated group within 4–5 days (Fig. 3C). These results suggest that the efficacy observed may reflect suppression by VE-13,045 of an active IL-1β dependent inflammatory process. The efficacy of VE-13,045 was next evaluated in animals with established CIA. Daily treatment (50 mg/kg) was initiated in randomized groups of animals with focal swelling of the wrist (level 2) starting 10 days after the CII booster injection. Mice treated with the ICE inhibitor showed a 20% reduction in inflammation beginning 2 days after treatment initiation, with a 40% overall reduction in disease severity that was sustained over the 16 day treatment interval (significance of $P < 0.01$; Fig. 3D). Progression of arthritic disease (from a combined score of 4–5; a 20% increase in severity) was observed in the vehicle-treated group.

Histological evaluation of wrist paw joints from untreated and VE-13,045 treated mice

Wrist paw joints from vehicle-treated and VE-13,045 treated mice were evaluated to assess the extent of synovitis, inflammatory cell infiltration, fibrosis, and cartilage erosion. Figure 4 A–F shows histological changes in wrist joints from representative mice. Compared to a normal mouse wrist joint (Fig. 4A), invagination of the synovial membrane with infiltration of inflammatory cells and marked cartilage erosion is evident in the wrist joint of a representative vehicle-treated mouse 23 days after the CII booster injection (Fig. 4B). In contrast, prophylactic treatment with VE-13,045 reduced the extent of synovial membrane damage and inflammatory cell infiltration and minimized cartilage erosion (Fig. 4C). In the therapeutic regimen, histological evaluation shows progression of arthritic disease with invagination of the synovial membrane, inflammatory cell infiltration, fibrosis and cartilage erosion in untreated mice (Fig. 4E) compared to the histological extent of joint damage noted in a representative mouse sacrificed 10 days after the CII booster (Fig. 4D). VE-13,045 treatment prevented further progression of inflammatory cell infiltration and joint destruction (Fig. 4F) concurrent with the reduction in inflammatory symptoms observed in these animals (see Fig. 3D).

Treatment with an ICE inhibitor reduces serum amyloid A levels

Sera were obtained from animals in untreated and treated groups at three time points during the clinical course of CIA for determination of serum amyloid A (SAA) levels. Table 2 shows that treatment with indomethacin and prednisolone reduced SAA levels compared to the vehicle-treated group within 5 days of the CII booster injection. VE-13,045 treatment (50 mg/kg/day) also reduced SAA levels suggesting that a generalized reduction in the acute phase inflammatory response is associated with ICE inhibition in CIA. Treatment with each of the three agents reduced SAA to a level comparable to normal mice (4.5 ± 0.3 μg/ml) by Day 26, independent of the clinical status of CIA at that time (Table 2).

TABLE 2. Time course profile of serum amyloid A levels in mice with Type II collagen-induced arthritis

	Time after type II collagen booster		
Treatment group	Day 5	Day 12	Day 26
Vehicle-treated	39.2 ± 25	21.3 ± 10.3	24.2 ± 7.6
VE-13,045 50 mg/kg/d	20.2 ± 3	11.9 ± 6	4.5 ± 1.2
Prednisolone 10 mg/kg/d	9.6 ± 6	8.0 ± 5.4	5.3 ± 1.8
Indomethacin 2 mg/kg/d	14.9 ± 6.4	3.4 ± 0.3	3.2 ± 0.2

Mice ($n = 8$ per treatment group) were bled at three time points during the clinical course of CIA and serum amyloid A levels were determined by ELISA. Data are expressed as the mean ± standard deviation in μg/ml. Serum amyloid A levels for normal DBA/1J mice ($n = 6$) were 4.5 ± 0.3 μg/ml.

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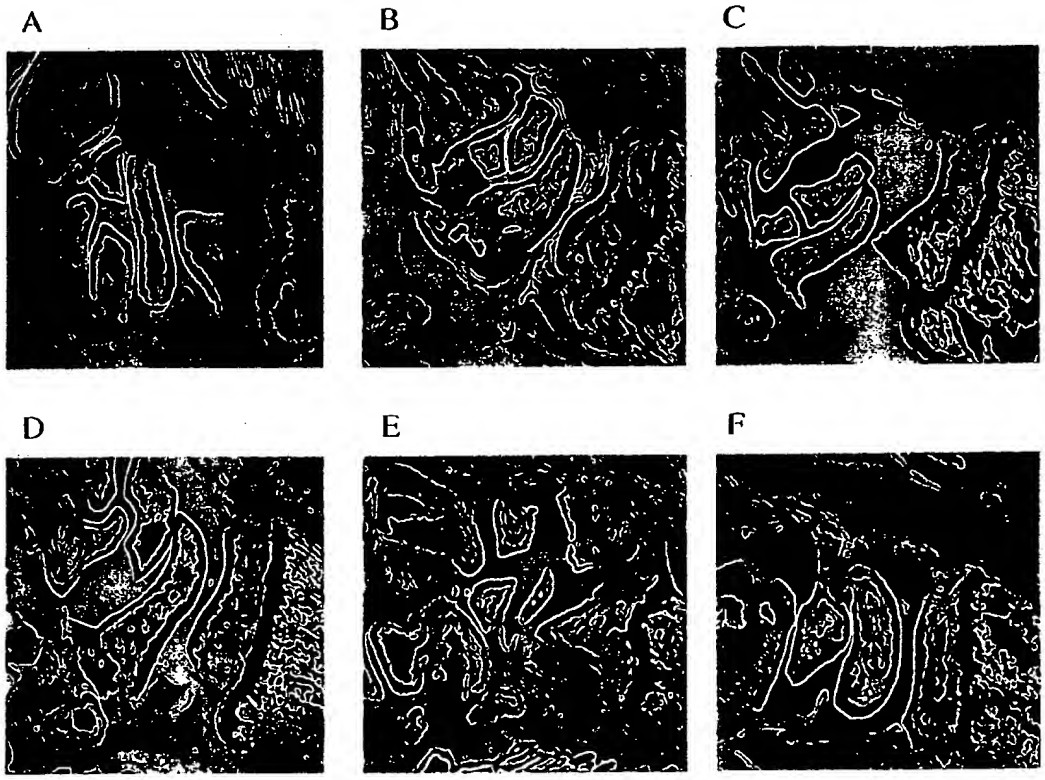

Figure 4. Histological evaluation of front paw wrist joints from representative DBA/1J mice with Type II collagen-induced arthritis.

(A–C) Prophylactic therapy of CIA: comparison of a wrist joint from a normal mouse (A) and a vehicle-treated mouse (B) on day 23 shows invagination of the synovial membrane with fibrosis and inflammatory cell infiltration into the joint space with cartilage erosion compared to a VE-13,045 wrist joint (C) on day 23 with minimal evidence of synovitis or cartilage erosion and no inflammatory cell infilatrate in the joint space. (D–F) Therapy of established CIA: a wrist joint 10 days after the CII booster injection (D) with initial evidence of synovitis and cellular infiltration compared to progression of CIA in an untreated wrist joint (E) on day 26, with extensive synovitis, fibrosis, cellular infiltration and complete erosion of cartilage over the bone growth plate. VE-13,045 treatment (F) prevented further progression of CIA evaluated on day 26 compared to the histologic extent of disease on day 10 (D).

DISCUSSION

We have shown that two irreversible prototype inhibitors of ICE effectively block secretion of both IL-1β and IL-1α in vitro and provide the first evidence for efficacy of an ICE inhibitor in a chronic inflammatory disease model. Murine CIA shares several immunological and pathogenetic features of RA in humans[33] and recent studies have identified a prominent role for IL-1, TNF-α and other proinflammatory cytokines and chemokines in the evolution of CIA.[39–43] Treatment of mice with IL-1RA has been shown to delay the onset and reduce the incidence and severity of CIA,[14] and prophylactic treatment with anti-IL-1β and anti-IL-1α antibodies alone or in combination can completely alleviate CIA in mice.[34,35] Similarly, prophylactic treatment with anti-TNF-α,[44] soluble TNF-α receptor[45] or a TNF-α receptor-Fc fusion protein[46] may also reduce the incidence and severity of CIA.

In addition to a role in the induction of CIA, localized production of IL-1β and TNF-α by synoviocytes and infiltrating inflammatory cells contributes to progressive disease and joint destruction. Both cytokines induce tissue degrading matrix metalloproteinases and may suppress chondrocyte proteoglycan synthesis. Administration of IL-1 to mice with antigen[47] or collagen-induced arthritis[48] exacerbates the course of disease due to enhanced synovitis, inflammatory cell infiltration, fibrosis, cartilage and bone erosion. Similar results have been observed after intra-articular injection of TNF-α[49] and mice expressing a human TNF-α transgene develop a chronic destructive polyarthritis that can be prevented by treatment with anti-TNF-α antibodies.[50] The multiple roles of IL-1 and TNF-α in arthritic disease[51] and efficacy observed with IL-1RA, anti-IL-1 or TNF-α antibody treatments suggests strategies for potential therapeutic intervention in both the induction and progressive phases of disease.

Results of this study implicate activation of ICE and subsequent processing of IL-1β in the induction and progression of CIA. Prophylactic treatment with VE-13,045 delayed the onset of inflammatory symptoms, blocked progression of synovitis and infiltration of inflammatory cells and reduced joint destruction in comparison to vehicle-treated animals. Therapeutic treatment with VE-13,045 also blocked progression of inflammation and halted further cellular infiltration and cartilage erosion. ICE inhibition in established disease appears to be more effective than blocking TNF-α since anti-TNF-α treatment is less effective in reversing the course of established disease.[44,45] The efficacy observed with VE-13,045 in both prophylactic and therapeutic treatment regimens is further evidence of the central role of IL-1β in CIA and our results also suggest that the CIA model is well suited for pharmacodynamic evaluation of ICE inhibitors.

VE-13,045 is remarkably effective in vivo considering its modest in vitro potency (IC_{50} ~1.3 μM for VE-16,084), C_{max} (10–12 μg/ml) and short plasma half life ($t_{1/2}\beta$ ~20 min). The efficacy observed with VE-13,045 may reflect in part the irreversible nature of this inhibitor due to inactivation of ICE molecules in cells at the inflammatory site. Compared to a reversible inhibitor, synthesis of new ICE precursor or processing of active ICE from existing p45 precursor in macrophages, synoviocytes or other IL-1β producing cells in situ is necessary to overcome the irreversible inhibitor effect. Inflammatory and arthritic symptoms and histological evidence of disease persisted in animals in both the prophylactic and therapeutic treatment groups. Sustained disease may result from less than complete inhibition of ICE activity, which is consistent with the single daily administration of VE-13,045 and its short half life. Alternatively, residual disease may reflect the complexity of the cellular and molecular components involved in the pathogenesis of CIA. In addition to IL-1 and TNF-α, other cytokines (IFN-γ, IL-6, TGF-β) and chemokines (MIP-1α and MIP-2) may contribute to joint inflammation.[39–43] Also, local deposition of anti-Type II collagen antibodies, complement activation, generation of C5a, and production of leukotrienes or other mediators may lead to recruitment of cells to the inflammatory site, sustaining an intermediate level of joint inflammation. However, the near complete suppression of CIA with anti-IL-1 antibody treatment[34,35] suggests that more frequent administration or higher doses of an ICE inhibitor, or the use of more potent compounds with improved pharmacokinetics may further enhance the efficacy of an ICE inhibitor in CIA.

The inhibition of IL-1α secretion by ICE inhibitors in vitro is consistent with results from studies with ICE-deficient mice. We and others[32,52] have observed diminished secretion of IL-1α by LPS stimulated monocytes from $ICE^{-/-}$ mice compared to monocytes from $ICE^{+/+}$ mice. Also, $ICE^{-/-}$ mice challenged with 32 mg/kg LPS had diminished serum levels of IL-1α (52; G. Ku and M. W. Harding, unpublished observations). Inhibition of IL-1α secretion by ICE inhibitors or in $ICE^{-/-}$ mice suggests a role for ICE in IL-1α release. Although ICE does not directly process the IL-1α precursor,[26] ICE may interact with or activate calpain[23,24] or other proteins involved in IL-1α processing and release. Alternatively, ICE may be part of a molecular assembly or complex involved in the export of IL-1α through the plasma membrane.

A recent study identified several variants of ICE (ICE-β, ICE-γ, ICE-δ and ICE-ε) generated by alternative splicing of ICE mRNA.[53] It is possible that ICE, or a splicing variant of ICE, may perform a chaperonin-like function in IL-1α release, independent of its protease activity. If the effect of VE-13,045 and VE-16,084 observed in this study result from steric hindrance or a conformational change in ICE that alters critical protein-protein interactions, it will be of interest to evaluate other chemical classes of ICE inhibitors for an effect on IL-1α release. A strategy for blocking the biological actions of IL-1α may result from further investigating the role of ICE in IL-1α release.

Improving on the irreversible, peptidyl ICE inhibitors for therapeutic applications will require the design of non-peptidyl compounds. The availability of the high-resolution crystal structure of ICE, as well as structure-activity investigations of peptidyl ICE inhibitors, should lead ultimately to the design of an orally bioavailable compound with improved cellular potency and pharmacokinetic properties. The efficacy of VE-13,045 in CIA suggests that such a compound may be suitable for clinical evaluation in patients with RA, osteoarthritis or other clinical indications where IL-1β contributes to the progression of inflammatory disease.

MATERIALS AND METHODS

Materials

Lipopolysaccharide (LPS, from *E. coli* serotype 0111:B4, trichloroacetic acid extracted), indomethacin, methyl prednisolone, Freund's complete adjuvant, and carboxymethyl cellulose were purchased from Sigma (St Louis, MO); chick sternum-derived Type II collagen (CII) from Elastin Products (Owensville, MO) and olive oil (extra virgin) from a local store. Male CD1 mice (20–22 grams) and male DBA/1J mice (age 6 weeks) were purchased from Charles River (Wilmington, MA) and Jackson Labs (Bar Harbor, ME), respectively. Mice were given food (Purina rodent chow) and water *ad libitum*. ELISAs for murine IL-1β (PerSeptive Diagnostics, Cambridge, MA), human IL-1α and IL-1β (R & D Systems, Minneapolis, MN), murine IL-1α (Genzyme,

Cambridge, MA), TNF-α, IL-6 and murine serum amyloid A (Biosource International, Camarillo, CA) were obtained from commercial sources and performed according to the manufacturers' suggested protocols. VE-13,045 and VE-16,084 were provided by Dr J. M. C. Golec (Hoechst-Roussel, Swindon, UK). Stock solutions were prepared in DMSO and stored at −20°C.

Induction of IL-1β and IL-1α in vitro

Buffy coat cells were obtained from blood donors and peripheral blood mononuclear cells (PBMC) were isolated by centrifugation in LeukoPrep tubes (Becton-Dickinson, Lincoln Park, NJ). PBMC were added (3×10^6/well) to 24-well Corning tissue culture plates and after 1 h incubation at 37°C, non-adherent cells were removed by gentle washing. Adherent mononuclear cells were stimulated with LPS (1 μg/ml) with or without ICE inhibitors (0.2–20 μM VE-13,045 or VE-16,084) in 2 ml RPMI-1640-10% FBS. After 16–18 h incubation at 37°C, IL-1β and IL-1α were quantitated in culture supernatants by ELISA. For isolation of murine adherent mononuclear cells, spleens were excised from DBA/1J mice, single cell suspensions were prepared and added to 12-well tissue culture plates (1×10^7/well), adherent mononuclear cells were isolated and stimulated with LPS (10 μg/ml), with or without ICE inhibitors in 0.5 ml RPMI-1640-10% FBS. After 16–18 h, supernatants were harvested, cells were washed once, incubated for 30 min with 10 μM nigericin[36] in 0.5 ml RPMI-1640-10% FBS containing the ICE inhibitors (at the same concentrations as in the overnight cultures), and supernatants were harvested again for quantitation of IL-1β and IL-1α. Viability of monocytes before or after nigericin stimulation was >98% as measured by trypan blue staining or lactate dehydrogenase activity in culture supernantants, indicating that minimal cytolysis had occurred during the experiments.

Acute induction of IL-1α and IL-1β production in vivo

LPS mixed with 0.5% carboxymethyl cellulose in PBS, pH 7.4, was administered by intraperitoneal injection (40 mg/kg LPS) in a dose volume of 20 ml/kg. VE-13,045 stock solutions in DMSO were then dissolved in olive oil:ethanol:DMSO (final concentration 90:5:5; v/v/v) and administered by intraperitoneal injection at 50 mg/kg in a dose volume of 5 ml/kg. VE-13,045 or the vehicle [olive oil:ethanol:DMSO (90:5:5, v/v/v)] alone was administered to mice simultaneous with LPS, or at various time points before or after LPS. Mice were euthanized 7 h after LPS challenge for blood collection. Serum IL-1β was measured with an ELISA specific for mature IL-1β. Specificity was determined by testing the reactivity of purified recombinant murine pro-IL-1β in the ELISA. At concentrations of 20 pg/ml and 10 ng/ml, respectively, mature and precursor IL-1β were recognized equally, indicating a cross-reactivity of 0.2% (data not shown). Serum IL-1α was measured with an ELISA that detects both the precursor and mature forms. Statistical significance between groups was determined by Student's *t*-tests.

Type II collagen-induced arthritis

Type II collagen-induced arthritis was established in male DBA/1J mice as described by Wooley.[37] Briefly, chick sternum Type II collagen (4 mg/ml in 10 mM acetic acid) was emulsified with an equal volume of Freund's complete adjuvant (FCA) by repeated passages (400) between two 10-ml glass syringes connected with a gauge 16 double-hub needle. Mice were immunized by intradermal injection (50 μl; 100 μg CII per mouse) of the collagen emulsion at the base of the tail on day 0 and again with 50 μl or 100 μl (200 μg CII) of a freshly prepared collagen emulsion 21 days later at the contra-lateral side of the tail base. VE-13,045 was prepared as described above (90:5:5 olive oil:ethanol:DMSO) and administered daily (25, 50 or 100 mg/kg) by intraperitoneal injection. Both indomethacin (2 mg/kg) and methylprednisolone (10 mg/kg) were prepared in PBS and administered daily by oral gavage. For the prophylactic regimen, drug treatments were initiated within 2 h of the CII booster immunization. For the therapeutic regimen, mice with a similar degree of inflammation were selected (10 days following the CII booster) and randomized for treatment with VE-13,045 or the vehicle alone.

Scoring of arthritic symptoms

Front paws (ventral surface) were examined daily after the CII booster injection and scored (Arthritis Index) for severity of inflammation and arthritic disease as follows: Level 1 - erythema; Level 2 - focal carpal (wrist) joint swelling; Level 3 - swelling of the entire wrist; Level 4 - spread of swelling to the metacarpal/metatarsal (palm) region; Level 5 - swelling affecting the metacarpophalangeal or the metatarsophalangeal joints. The score of both front paws was combined and the mean ± SEM determined for each treatment group. Rear paws were not scored in this study. The statistical significance between groups was determined by Mann-Whitney nonparametric analysis.

Histological examination of inflamed paws

Untreated animals with arthritic disease at each severity level were sacrificed and paws were removed for histological evaluation. Animals in prophylactic regimens were sacrificed at the end of the treatment period. For the therapeutic regimen, animals exhibiting comparable levels of inflammation were sacrificed on day 10 and front paws were processed for histological evaluation as a reference. The remainder of the animals were sacrificed at the end of the treatment period. Paws were fixed with 10% formalin in PBS for 48 h at 25°C, then de-calcified in 10% formic acid in water for 24 h at 25°C. The tissues were embedded in paraffin, sagital sections were prepared, and stained with Giemsa.

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